REMARKS

I. Introduction.

Claims 1-32 were examined. Claims 33-43 have been canceled, as drawn to the nonelected invention. The cancellation of claims is done without prejudice to further prosecution in a divisional application.

Claims 1-25, 27-32 and 44 are pending. Claims 1, 14, 19, 20, 21, 24, 25, 29, 30, and 32 are amended. These claims are amended to more specifically claim certain embodiments of the instant invention. The invention, as now more specifically claimed with the amendments, is directed to targeting activated MLK activity in neuronal cells with a compound to determine that compound's ability to prevent neuronal cell death. The amendments of the claims are done without prejudice to further prosecution of other embodiments of this invention in a continuation, continuation-in-part, divisional, or other related application.

This invention is based on the discovery that expression of MLK activates the SEK1-JNK pathway and induced apoptosis in neuronal cells. Thus, over-activation or stimulation of the MLK-SEK1-JNK cascade mediates neuronal toxicity. Since over-activation of glutamate receptor induces excitotoxicity in neurons and is a common pathway responsible for neuronal death in a variety of neurodegenerative diseases and in various acute insults such as hypoxia, ischemia, stroke, and others, the over-activation of the MLK-SEK1-JNK pathway serves as a common molecular mechanism for neuronal loss in these illnesses. Therefore, inhibition of the activation of the MLK-SEK1-JNK pathway by suppressing MLK-associated activities will prevent neuronal death in these neurological diseases. Prior to Applicant's invention, there was no report on the role of MLK activation in any kind of neuronal toxicity.

II. Priority Application

The Examiner states that Applicant is given the benefit of the filing date of September 17, 1998, and is being denied benefit to the provisional application filing date of May 14, 1998. According to the Examiner the basis for this decision is that "the full scope embraced by each claim was not disclosed in the provisional application."

Applicant respectfully submit that she is entitled to priority to the May 14, 1998, date for as much as what is disclosed and fully supported by the provisional application.

The priority application provides support for a method of screening or assessing a compound's ability to prevent neuronal cell death by containing a compound with neuronal cells having both activated JNK or MLK activity. The invention, as now claimed, is directed to MLK activity. The Examiner's attention is directed to the provisional patent application at the following pages which provide support for MLK activity:

Page 2, lines 8-15:

Moreover, normal huntingtin is associated with MLK2, a nuclear kinase which is almost exclusively expressed in brains. Expression of MLK2 also activated the SEK1-JNK pathway and induced apoptosis in HN33 cells. Co-expression of mutated huntingtin with MLK2 induced apoptosis in 293 cells while expression of normal or mutated huntingtin or MLK2 alone, or co-expression of normal huntingtin with MLK2 did not generate any toxic effect. Taken together, our studies demonstrate that expression of polyglutamine-expanded huntingtin induces neuronal apoptosis by activation of the SEK1-JNK pathway and this effect may be mediated by MLK2.

Page 5, last paragraph to page 7, line 16:

We then investigated the signal transduction pathway involved in activation of the SEK1-JNK pathway. In a previous study, we demonstrated that huntingtin interacts with SH3 domain-containing proteins such as Grb2 and RasGAP (18).

Members of the MLK family are the only known SH3 domain-containing proteins which activate JNKs (19-21). MLKs directly bind and activates [*sic* activate] SEK1 which in turn elevates the JNK activity. Since MLK2 is a neuronal form of MLKs, we examined the effect of MLK2 expression in HN33 cells. NO any [*sic* No] toxic effect was observed in HN33 cells transfected with pRK5CMV vector (Fig. 4A, control). Expression of MLK2 caused apoptotic cell death in HN33 cells and co-expression of dominant negative SEK1 mutant (SE1K/R) attenuated MLK2-mediated neuronal apoptosis (Fig. 4A). Dominant negative SEK1 was clearly more effective at inhibiting cell toxicity induced by mutated huntingtin than by MLK2. This observation is consistent with other reports (20-21) and reflects the fact that MLK2 is a constitutively active kinase whose action is more difficult to block although SEK1 is direct downstream of MLK2. Mutated huntingtin, on the other hand, lacks enzyme activity and may regulate activity of endogenous MLK2 in HN33 cells. Therefore, its action on the SEK2-JNK pathway may be more easily attenuated. Taken together, these results demonstrate that MLK2, like mutated huntingtin, also activates the SEK1-JNK pathway to induce cell death in HN33 cells.

Next, we explored the potential association of huntingtin with MLK2. Because expression of either mutated huntingtin or MLK2 alone cause rapid cell death in HN33 cells (Table 1), we could not obtain sufficient cells to perform a co-immunoprecipitation experiment. No toxic effect was observed in 293 cells transfected or co-transfected with control vectors (Table 1). Expression of MLK2, or normal or polyglutamine-expanded huntingtin alone in 293 cells also did not generate cell toxicity at 48 hours after transfection (Table 1) and expression of normal or mutated huntingtin failed to activate JNKs (data not shown). Since 293 cells are rich in huntingtin (18), we examined the interaction of MLK2 with normal huntingtin in 293 cells. c-Myc tagged MLK2 was transiently expressed in 293 cells and MLK2 was precipitated with anti-c-myc tag 9E10 antibody (22). In cell lysates from 293 cells transfected without (Fig. 4B, 9E10IPC) or with pRk5CMV vector alone (Fig. 4B, 9E10IPV), 9E10 failed to co-precipitate huntingtin (Fig. 4B), while huntingtin was easily detected in both 9E10 (Fig. 4B, 9E10IPT) and anti-huntingtin (Fig. 4B, HDPIP) immunoprecipitates of cell lysates from 293 cells transfected with c-myc tagged MLK2. Conversely, we determined whether an anti-huntingtin antibody precipitates MLK2. Cell lysates from 293 cells with or without transfection of MLK2 were incubated with 437, an anti-huntingtin antibody or 9E10. In non-transfected wild-type 293 cells, MLK2 was not detectable in both 9E10 and 437 immunoprecipitates (Fig. 4C, 9E10IPC) and in pRK5CMV transfected cells, MLK2 was also absent in 9E10 immunoprecipitates (Fig. 4C, 9E10IPV). Whereas in MLK2 transfected 293 cells, MLK2 was detected in both 9E10 and 437 immunoprecipitates (Fig. 4C, 9E10IPT

& 437IPT). Since the detected association of MLK2 with endogenous huntingtin only takes place in MLK2 transfected 293 cells, not in wild-type 293 cells or 293 cells transfected with pRK5CMV expression a c-myc tag alone, these studies suggest that normal huntingtin interacts with MLK2 in intact cells. Next we attempted to examine a potential difference between normal and mutated huntingtin interaction with MLK2. Co-transfection of pFL16HD, which expresses normal huntingtin with a 16 CAG repeats, with MLK2 vector did not produce any cell toxicity (Table 1). Although expression of mutated huntingtin or MLK2 alone did not influence 293 cell vitality (Table 1), co-expression of mutated huntingtin with MLK2 induced rapid apoptosis and most cells died within 48 hours after transfection (Table 1). Thus, we could not obtain enough 293 cells co-transfected with MLK2 and mutated huntingtin to perform [a] co-immunoprecipitation experiment to determine any alteration of the interaction of MLK2 with polyglutamine repeat-expanded huntingtin. However, these results do suggest that MLK2 and mutated huntingtin synergistically stimulate the apoptosis signaling pathway in 293 cells."

Page 8, last paragraph to Page 9, line 16:

Several observations in our study suggest that MLK2 may be in involved mutated huntingtin-induced neuronal apoptosis. First, MLK2, like mutated huntingtin, initiates apoptotic cell death in HN33 cells. Second, mutated huntingtin, like MLK2, induces JNK activation. Third, neuronal toxicity induced by either mutated huntingtin or by MLK2 could be attenuated by dominant negative SEK1 which specifically inhibits JNK activation. Fourth, co-expression of normal huntingtin with MLK2 does not induce apoptosis in 293 cells while co-expression of mutated huntingtin with MLK2 results in cell death. Furthermore, MLK2 is present in HN33 cells and absent in 293 cells according to a RT-PCR analysis (data not shown). Thus, mutated huntingtin-mediated cell toxicity requires the presence of MLK2. The precise mechanism for how MLK2 is directly involved in mutated huntingtin-mediated neuronal apoptosis is not clear. Since normal huntingtin is associated with MLK2 in intact cells and such an association does not generate any cell toxicity, it may be possible that normal huntingtin is an inhibitor of MLK2 while expansion of the polyglutamine repeat in huntingtin interferes with its association with MLK2 and leads to activation of the SEK1-JNK pathway. As a result, mutated huntingtin may act as a dominant negative mutant and attenuate the inhibitory action of normal huntingtin on the MLK2-SEK1-JNK pathway. This may explain why HD is inherited in a dominant fashion. Currently, we are further investigating how MLK2 is involved in mutated huntingtin-mediated neuronal toxicity. In addition to huntingtin, most

> proteins involved in CAG-expanded hereditary diseases, such as ataxin 1, ataxin 2, ataxin 6, ataxin 7, DRPLA, and androgen receptor (27), all contain one or more potential SH3 domain binding motifs and potentially , they may all bind to MLK2. Thus, activation of the MLK2-SEK1-JNK pathway may be one of common molecular mechanisms for neuronal loss in CAG-expansed neurodegenerative diseases.

The Examiner's attention is also directed to Figure 4 and Table 1 of the provisional application which provides enabling support as required under 35 U.S.C. § 112, first paragraph, for the claims of the instant application.

The instant application is admittedly an application that contains additional information to that of the provisional application. Applicant submits that the provisional application fully supports and enables the instant application. Further, this situation is analogous to that of filing a continuation-in-part application from an earlier filed application. As stated in MPEP ¶ 706.02, page 700-10, any claims of the cip application which are fully supported under § 112 by the earlier parent application will have the effective filing date of that earlier parent application.

III. Rejection Under Section 102(a) based on Liu et al., Society for Neuroscience Abstracts, October 1997.

Claims 1-2, 4, 6, 8-9, 11, 13-15, and 17 are rejected under 35 U.S.C. § 102(a) as anticipated by Liu et al., Society for Neuroscience Abstracts, October, 1997. According to the Examiner, Liu et al is cited as it is authored by other people in addition to the named inventor. In addition, the Examiner states that "Liu et al. teaches that expression of the huntingtin mutant activates JNK/APK and induces neuronal apoptosis in hippocampal cells. Dominant-negative SEK(K-r) inhibits this induced apoptosis and may be a therapeutic tool in Huntingtin's Disease."

Applicant is submitting with this Amendment an *In re Katz* type declaration under 37 C.F.R. §1.132. With the filing of this declaration, this rejection should now be moot.

III. Rejection Under Section 103(a) based on Liu et al., Society for Neuroscience Abstracts, October 1997.

Claims 9-10, 14, and 18 are rejected under 35 U.S.C. § 103(a) as obvious over Liu et al., Society for Neuroscience Abstracts, October, 1997.

With the filing of the *In re Katz* declaration under Rule 132, it is submitted that this rejection is now moot.

IV. Rejection Under Section 103(a) based on Yardin, et al, Neuroreport, (June 22, 1998), Ni et al, U.S. 5,840,509, and Johnson, U.S. 5,854,043.

Claims 1-2, 5-9, 12-16, and 19-32 are rejected under 35 U.S.C. § 103(a) as obvious over the combination of Yardin, et al, Neuroreport, June 22, 1998, Ni et al, U.S. 5,840,509, and Johnson, U.S. 5,854,043. According to the Examiner, "It would have been obvious to use the experimental system of Yardin et al. to assess a compound's ability to prevent neuronal cell death in neurological conditions. . . . Johnson, Yardin et al., and Ni et al. make clear that it would have been well known how to manipulate various aspects of the second messenger systems to evaluate inhibitors and induces of apoptosis, enzymatic activity, gene expression, and so forth in the cascade, using well known techniques."

Applicant respectfully traverses this rejection.

To begin, Yardin et al. is a reference with a date as of June 22, 1998. Applicant is entitled to a priority date of May 14, 1998. For that reason, Yardin et al. is not an appropriate reference.

Even if Yardin et al. were an appropriate reference, Yardin et al. does not render obvious the invention, as now claimed, obvious.

Yardin et al. demonstrate that FK506, an immunosuuppressive drug, can block neuronal apoptosis induced by serum deprived cortical (neuronal) cell cultures. The results showed that the FK506 prevented the expression of the C-jun protein in these serum deprived (stressed) neuronal cell cultures. Yardin et al. state that "The links between the association of FK506-FKBP12 and C-jun are not known but could implicate ATF-2 in the induction of C-jun transcription. Further work will be needed to analyze the relationship between C-jun, ATF-2 and FK506 since ATF-2 [activating transcription factor-2] is also a substrate for the C-jun kinases which phosphorylate C-jun protein." (Yardin et al, page 2080, last paragraph.)

c-Jun is a transcription factor and its expression can be influenced or regulated by a number of factors, including JNK phosphorylation and in some cell types by ERK-mediated mechanisms. (See the review article, Leppa and Bohmann, "Diverse functions of JNK signaling and c-Jun in stress response and apoptosis," *Oncogene* 18:6158-6162 (1999)and Leppo et al., "Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation," *EMBO Journal* 17:4404-4413 (1998). Copies of these articles are enclosed for the convenience of the Examiner.) Thus, as Yardin et al. demonstrate, other factors influence the expression of c-Jun.

Yardin et al. does not recognize, much less render obvious, Applicants invention as now claimed, namely, that the inhibition of MLK activities in the MLK-SEK1-JNK pathway will prevent neuronal death in these neurological diseases.

Ni et al. is directed to a neuronal interleukin-1 converting enzyme (ICE) related protease and related DNA compounds and the use of this protease and its DNA in a method to identify compounds that inhibit the apoptotic process. Ni et al. states that

"ICE related protease is present in the CNS and is enriched in central neurons including pyramidal neurons and granule neurons of the hippocampus and cerebral cortex. Data suggests that overexpression of ICE related protease could be involved in a neuronal death cascade in mammalian neurons. A further understanding of the cellular events underlying apoptosis will probe useful for developing neuroprotective strategies as well as therapeutic interventions for head traumas, ALS, Alzheimer's stroke, brain ischemia, as well as a variety of other neurodegenerative disorders involving apoptosis." Col. 7, lines 43-53.

Applicant agree with the Examiner that it was known "how to manipulate various aspects of the second messenger systems to evaluate inhibitors and induces of apoptosis, enzymatic activity, gene expression, and so forth in the cascade, using well known techniques." Applicants disagree, however, that the combination of references teaches one of skill in the art that MLK can be used as a target for the development of inhibitory compounds of MLK-associated activity.

Johnson teaches a method for regulating the homeostasis of a cell by regulating the signal transduction activity of a mitogen extracellular signal-regulated kinase (ERK) kinase kinase protein (MEKK)-dependent pathway. Johnson discusses various ways in which the homeostasis of a cell is controlled by regulating the activity of an MEKK-dependent pathway in which the MEKK protein regulates the pathway substantially independent of Raf. Johnson also discusses regulating MEKK-dependent pathway by "contacting a cell with a compound capable of directly interacting with a protein including MEKK, JEK, JNK, Jun, ATF-2, and Myc, and combinations thereof, in such a manner that the proteins are activated; and/or contacting a cell with a compound capable of directly interacting with a protein including Raf, MEK, MAPK, TCF protein and combinations thereof in such a manner that the activity of the proteins are inhibited."

While Johnson does discuss increasing or decreasing the activity of JNK or Jun, it does not suggest to one of skill in the art that MLK can be used as a target for the development of inhibitory compounds of MLK-associated activity, and that such compounds can be used to prevent neuronal loss.

The combination of the cited references, Yardin et al., Ni et al., and Johnson does not render obvious Applicant's invention as now claimed. The claims, as now amended, are specifically directed to assessing or screening for a compound's ability to prevent neuronal cell death by contacting a compound with neuronal cells having activated MLK activity and determining by comparison the compound's ability to prevent cell death by the number of neuronal cells that die.

V. ***Rejection Under Section 103(a) based on Cheung et al, Journal of Neuroscience Research, (April 1, 1998), Ni et al, U.S. 5,840,509, and Johnson, U.S. 5,854,043.***

Claims 1-2, 5-7, 9, 12, 14-16, 19-22, and 24-32 are rejected under 35 U.S.C. § 103(a) as obvious over the combination of Cheung et al, Journal of Neuroscience Research, (April 1, 1998), Ni et al, U.S. 5,840,509, and Johnson, U.S. 5,854,043. According to the Examiner, "it would have been obvious to use the experimental system of Cheung et al. to assess a compound's ability to prevent neuronal cell death in neurological conditions. Apoptosis would have been well known to be involved in neurological conditions and the prior art teaches the association between c-Jun and apoptosis. Johnson, Cheung et al., and Ni et al. make clear that it would have been well known how to manipulate various aspects of the second messenger systems to evaluate inhibitors and induces of apoptosis, enzymatic activity, gene expression, and so forth in the cascade, using well known techniques."

Applicant respectfully traverses this rejection.

Cheung et al. studied the relationship between the expression of c-Jun and kainate-induced cell death in cerebellar granule cells. Cheung et al. states that kainate-induced cell death correlated with an increase in c-Jun mRNA and suggest that c-Jun expression is activated by apoptotic stimuli and is a potential marker for apoptosis. (See Cheung et al., page 78, second column, first paragraph.)

However, Cheung et al. does not teach nor suggest to one of skill in the art that MLK can be used as a target for the development of inhibitory compounds of MLK-associated activity. Even with the combination of Ni et al. and Johnson et al., one of skill in the art would only be left with the general suggestion of manipulating cellular pathways to evaluate inhibitors of apoptosis. This general suggestion is an invitation to invention, and does not render the claimed invention obvious.

VI. Conclusion.

Applicants respectfully submit that all the basis for rejection of the pending claims are now moot. The Examiner is requested to reconsider the rejections and to withdraw them and to pass this case to issuance.

Respectfully submitted,

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Diverse functions of JNK signaling and c-Jun in stress response and apoptosis

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c-Jun/AP-1 activation has been implicated in various, often opposing cellular responses. For example, although there is considerable evidence that c-Jun activation can be a positive step in the events leading a cell towards apoptosis, there are also many reports stating the opposite: that under certain circumstances c-Jun can inhibit apoptosis and promote proliferation or differentiation instead – and that these responses are important for normal mammalian development. It is clear that the effects of c-Jun on cellular responses depend strongly on the cell type and the context of other regulatory influences that the cell is receiving. This review focuses on recent developments in understanding how activation of JNK and c-Jun contributes to different cellular responses.

Keywords: c-Jun; JNK; signal transduction; apoptosis

Introduction

The AP-1 family represents a paradigm for signal-responsive transcription factors. Many of the properties and regulatory functions of eukaryotic transcriptional regulators were initially discovered and described in studies on these proteins (Angel and Karin, 1991; Curran and Franza, 1988). Similarly, several currently unresolved questions in the field of signal transduction and gene regulation are addressed using the comparatively well-understood AP-1 family as an example. Such questions include: how are different extracellular signals integrated by complex networks of cellular information flow, and how are different incoming signals interpreted by the cell in a manner that takes the context into account, so that sensible and biologically appropriate responses are initiated. The role of c-Jun/AP-1 in stress response and the control of cell growth and apoptosis represents a good example for such complexities. Here we review some recent findings that illustrate the multi-facetted functions of c-Jun in the control of these cellular responses. For a more comprehensive review on the role of AP-1 factors in MAPK signal transduction see (Ip and Davis, 1998; Karin, 1995; Whitmarsh and Davis, 1996).

The AP-1 family consists of several groups of bZIP-domain (bZIP = basic region leucine zipper) proteins: the Jun, the Fos, and the ATF-2 subfamilies (Angel and Karin, 1991). Mammalian Jun proteins include c-Jun, JunB, and JunD; Fos proteins are c-Fos, FosB, Fra-1 and Fra-2; and the ATF proteins that are customarily included in the AP-1 family are ATF-2 and ATF-a. Like all bZIP transcription factors, AP-1 proteins have to dimerize before they can bind to their DNA target sites identified by the sequences TGACTCA, TGACGTCA, or variants thereof. Dozens of different homo- and heterodimeric combinations with different regulatory properties, as determined by the characteristics of the subunits, can form. The activity of individual AP-1 components can be regulated at different levels. One level is transcriptional. Some AP-1-encoding genes are tightly regulated: c-*jun* and c-*fos* are the best-characterized examples of this group. Their expression is subject to regulation by a large number of stimuli and signaling pathways (Whitmarsh and Davis, 1996). Other AP-1 coding-genes, such as *junD* and *ATF-2*, are expressed at fairly constant levels (de Groot *et al.*, 1991; Gupta *et al.*, 1995; Hirai *et al.*, 1989; Van Dam *et al.*, 1995).

In addition to the intracellular concentrations of these proteins, the specific activity of AP-1 factors is subject to regulation at the protein level, by post-translational modifications and interactions with other proteins. The mitogen-activated protein kinase (MAPK) signaling pathways play a predominant role in this regard (Ip and Davis, 1998; Karin *et al.*, 1997; Whitmarsh and Davis, 1996). These pathways are characterized by modules composed of three protein kinases: MAPKKKs phosphorylate and thereby activate MAPKKs, which in turn phosphorylate MAPKs. The three best-characterized subfamilies of MAPKs are named ERK, JNK and p38. In general, different MAPKs are members of separate modules and are regulated by distinct extracellular stimuli. For example, ERKs are activated by receptor tyrosine kinases and relay proliferation or differentiation signals. JNK and p38-type MAPKs are activated predominantly by stress stimuli and pathogenic insults, but in some cell types also by mitogens. Interestingly, all three classes of MAPKs are involved in the regulation of distinct AP-1 components. c-Jun is regulated by JNK phosphorylation and in some cell types also by ERK-mediated mechanisms. c-Fos is a substrate for regulatory phosphorylations by ERK, and ATF-2 is regulated by JNK and p38 kinases (Karin, 1995; Whitmarsh and Davis, 1996).

Reflecting this baffling complexity of regulatory inputs impinging on AP-1 factors, the range of biological responses in which these factors have been implicated is very broad. Signaling by AP-1 transcrip-

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tion factors has been shown to be involved in, or at least correlated with, phenomena as diverse as cell proliferation, transformation, different types of cell differentiation, cell migration and apoptosis. This review focuses on recent developments in understanding how activation of JNK and c-Jun/AP-1 contributes to different cellular responses.

c-Jun and cell proliferation

A role of c-Jun in growth control was first suggested by its ability to transform cells alone or in the presence of a cooperating oncogene (Bos *et al.*, 1990; Castellazzi *et al.*, 1991; Johnson *et al.*, 1996; Lloyd *et al.*, 1991; Schütte *et al.*, 1989). In addition, microinjection of antibodies against c-Jun was shown to inhibit progression of cells from G1 into S phase (Kovary and Bravo, 1991). Studies employing fibroblasts and/or hepatoblasts from *c-jun* knockout mice demonstrated that c-Jun deficiency results in a severe proliferation defect, which cannot be compensated by addition of purified mitogens (Eferl *et al.*, 1999; Hilberg *et al.*, 1993; Johnson *et al.*, 1993). Thus, at least in cultured fibroblasts and hepatoblasts, c-Jun acts as a positive regulator of cell growth.

Even though the molecular mechanisms underlying c-Jun-dependent growth control have not completely been resolved yet, one plausible mechanism by which c-Jun could positively regulate cell cycle progression was described recently and found to involve the tumor suppressor gene p53 (Schreiber *et al.*, 1999). In 3T3 fibroblasts lacking c-Jun, expression of p53 and its target gene, the cyclin-dependent kinase inhibitor p21, is upregulated resulting in impaired cyclin D1 and E-associated kinase activities, and inefficient exit of cells from G1 to S phase. It was demonstrated that in fibroblasts, c-Jun acts as a direct negative regulator of p53 expression. Consistently, deletion of p53 in *c-jun*−/− cells is sufficient to overcome all cell cycle and proliferation defects. In contrast to various stress stimuli, which upregulate p53 levels through a post-translational mechanism, c-Jun rather represses p53 expression at the transcriptional level by directly interacting with the p53 promoter. It is interesting to note that one of the mechanisms reported in the regulation of p53 at the protein level involves JNK, which has been proposed to down-regulate p53 by targeting it for degradation when the JNK signaling pathway is off, i.e. JNK is not catalytically active. Overexpression of c-Jun (Fuchs *et al.*, 1998a) or activation of JNK (Fuchs *et al.*, 1998b) leads to stabilization and accumulation of p53 in mouse fibroblasts. Thus, two components of the JNK pathway, JNK itself and c-Jun, can exert opposing effects on p53. This is puzzling at first, but may be explained by cell type differences. Furthermore, it is not clear yet whether the putative transcriptional repression of the p53 gene by c-Jun is phosphorylation-dependent, i.e. whether JNK or another MAPK plays a role in this process.

In a separate study, mouse embryo fibroblasts were also found to respond to loss of c-Jun by arresting in G1 phase (Wisdom *et al.*, 1999). However, in this cellular context, inhibition of cell proliferation is associated with reduced expression of cyclins D1 and D3. Importantly, cyclin D1 promoter activity was shown to be directly controlled by c-Jun. Together, these results establish a molecular link between c-Jun-dependent mitogenic signaling and cell cycle regulation.

Whether the activity of the JNK pathway and the c-Jun-mediated proliferation response are connected awaits further clarification. Two recent studies using gene targeting suggest that the Jun phosphorylation and/or the JNK pathway is involved. First, fibroblasts, in which endogenous *c-jun* gene is replaced by a mutant *c-jun* allele with the MAPK phosphoacceptor serines 63 and 73 changed to nonphosphorylable alanines (Ser63Ala, Ser73Ala), have a proliferation defect in comparison to wild type fibroblasts (Behrens *et al.*, 1999). Second, *sek1*-deficient fibroblasts also grow more slowly than their wild type counterparts (Ganiatsas *et al.*, 1998). However, in a separate study, which applied recombinant retroviruses directing the expression of different c-Jun mutants to rescue the proliferation defect in *c-jun*−/− cells, the phoshorylation sites, serines 63 and 73, were found not to be required (Wisdom *et al.*, 1999). In this context, it is also worth noting that the positive role of JNK pathway and c-Jun in proliferation appears not to be universal but rather a cell-type dependent phenomenon. Unlike in fibroblasts and hepatoblasts, at least in *sek*−/− and *c-jun*−/− embryonic stem cells, proliferation rates are not affected (Ganiatsas *et al.*, 1998; Hilberg and Wagner, 1992). Therefore, the net-effect of JNK and c-Jun activation depends on the cell context and, presumably, the signaling pathways that are simultaneously activated.

In addition to cell proliferation, c-Jun/AP-1 has been shown to play a role in differentiation. Studies in cell culture have indicated that c-Jun expression can promote differentiation of many different cell lineages, such as myeloid, neuronal and epithelial cells (Leppä *et al.*, 1998; Lord *et al.*, 1993; Szabo *et al.*, 1994). *In vivo*, c-Jun expression is required for viability (Table 1). Mice lacking c-Jun survive only to embryonic day 12 and die due to massive hemorrhage in the liver. A detailed histological analysis of *c-jun*−/− mice revealed that hepatoblasts and erythroid cells in the liver undergo prominent apoptosis (Eferl *et al.*, 1999). In addition, it was found that *c-jun* deficiency results in defects in the heart outflow track formation, a malformation resembling congenital human heart defect of a persistent truncus arteriosius (Eferl *et al.*, 1999). With respect to abnormal hepatogenesis, the *sek1*−/− phenotype is similar to *c-jun* deficient embryos (Ganiatsas *et al.*, 1998; Nishina *et al.*, 1999). In contrast, mutant mice lacking either JNK1, or JNK2, or JNK3 develop without obvious structural abnormalities. However, JNK1 as well as JNK2 mutant mice exhibit decreased activation-induced T-cell death and imbalance between T_H1 and T_H2 mediated immune responses (Dong *et al.*, 1998; Sabapathy *et al.*, 1999; Yang *et al.*, 1997b, 1998). In *Drosophila*, evidence has been presented for an involvement of Jun in several developmental processes. It has been shown that *Drosophila* Jun can induce photoreceptor differentiation after being phosphorylated by *Drosophila* ERK (Bohmann *et al.*, 1994; Peverali *et al.*, 1996) and that it acts as an effector of JNK signaling in the regulation of morphogenetic cell-

shape changes (Kockel *et al.*, 1997; Riesgo-Escovar and Hafen, 1997).

Jun as a mediator of apoptosis

Although, at first glance, cell proliferation and apoptosis appear to be opposing and mutually contradictory processes, the regulatory systems that control cell growth and cell death are remarkably overlapping. Indeed, a number of proteins that were originally identified as oncogene products and positive growth regulators were subsequently found to play important roles in apoptosis. Examples include Myc and Ras. In the case of c-Jun, ample evidence also exists that links the transcription factor to the control of cell death. However, the function of c-Jun in apoptosis control is rather multi-facetted, and represents a prime example for individual signaling components eliciting different or even opposing signal responses in different cellular settings.

Perhaps the most illustrative data for c-Jun acting as an inducer of apoptosis have been obtained in studies on neuronally differentiated cells. Initial studies using PC12 cells and sympathetic neurons showed that inhibition of c-Jun activity, either by microinjection of antibodies against c-Jun or expression of dominant negative mutant forms of the protein protects the cells from nerve growth factor (NGF) withdrawal-induced apoptosis (Estus *et al.*, 1994; Ham *et al.*, 1995; Xia *et al.*, 1995). Furthermore, ectopic expression of c-Jun is sufficient to drive sympathetic neurons into apoptosis in the absence of external stimuli (Ham *et al.*, 1995). Similarly, overexpression c-Jun was shown to induce apoptosis in 3T3 fibroblasts (Bossy-Wetzel *et al.*, 1997). Later studies revealed that, in addition to enhanced c-Jun expression, phosphorylation of c-Jun by JNK is necessary for the apoptotic response in certain neuronal cell types, including cerebellar granule and sympathetic neurons (Le-Niculescu *et al.*, 1999; Watson *et al.*, 1998). The most conclusive evidence for the importance of JNK activation and c-Jun phosphorylation for neuronal apoptosis in the animal has been obtained in gene targeting experiments (Table 1). It was shown that deletion of brain-specific *jnk3* gene, but not *jnk1* or *jnk2*, causes protection of hippocampal neurons from kainate-induced apoptosis (Dong *et al.*, 1998; Yang *et al.*, 1997b, 1998). Simultaneously, AP-1-dependent reporter activity, but not the activation of the c-*fos* and c-*jun* genes by kainate, is greatly reduced in the *jnk3* mutant background, suggesting that the post-translational regulation of AP-1 activity by JNK3 is critical for the triggering of death. In a pleasingly complimentary set of experiments, Wagner and collaborators showed that mice harboring a mutation in the c-*jun* locus that removes a subset of JNK phosphorylation sites (Ser63Ala, Ser73Ala) are also protected from kainate-induced apoptosis in the hippocampus (Behrens *et al.*, 1999). The mechanism by which JNK3 and c-Jun promote apoptosis remains obscure. A candidate effector is Fas ligand, which is induced in response to NGF withdrawal and JNK activation in PC12 cells (Le-Niculescu *et al.*, 1999). Other mediators of JNK signaling during apoptosis could be p53 and the apoptotic protein Bax (Aloyz *et al.*, 1998).

It has to be stressed that the essential positive role of c-Jun documented in hippocampal neurons exposed to kainate stress is not a reflection of a universal role of c-Jun in cell death or even in neuronal cell death. Mice

Table 1 Data from gene targeting experiments imply both positive and negative contributions of c-Jun and JNKs in the regulation of cell growth and apoptosis

	Embryonic phenotype of −/− mice	*Regulation of cellular response* *Apoptosis*	*Proliferation/ differentiation*	*Reference*
c-Jun	Lethal	Protection	Positive	(Behrens *et al.*, 1999; Eferl *et al.*, 1999; Hilberg *et al.*, 1993; Johnson *et al.*, 1993; Schreiber *et al.*, 1999; Wisdom *et al.*, 1999)
	Impaired hepatogenesis	UV	Fibroblasts Hepatoblasts	
	Defects in heart outflow track formation	Enhancement Glutamate toxicity in neurons		
c-JunAA 'knock-in'	Viable Decreased body weight	As above	As above	(Behrens *et al.*, 1999)
Jnk1	Viable	Enhancement Activation-induced T_H cell death	Positive T_H 1 effector cell differentiation	(Dong *et al.*, 1998)
Jnk2	Viable	As above	As above	(Sabapathy *et al.*, 1999; Yang *et al.*, 1998)
Jnk3	Viable	Enhancement Glutamate toxicity in neurons		(Yang *et al.*, 1997b)
Jnk1/Jnk2	Lethal Hindbrain exencephaly	Protection Forebrain Enhancement Hindbrain		(Kuan *et al.*, 1999)
Sek1	Lethal Impaired hepatogenesis	Protection CD95, CD3 and TCR-mediated thymocyte apoptosis	Positive CD 28-mediated T cell proliferation	(Ganiatsas *et al* 1998; Nishina *et al.*, 1996a,b, 1998, 1999; Yang *et al.*, 1997a)

lacking the c-Jun phosphorylation sites Ser 63 and 73 show no defects in several forms of normal developmentally regulated apoptosis (Behrens *et al.*, 1999). Even in the complete absence of c-Fos and c-Jun in double knockout mouse embryos, no defects in the normally occurring developmentally programmed cell death were recorded (Roffler-Tarlov *et al.*, 1996). Consistently, mutant mice deficient in either the *jnk1*, *jnk2*, or *jnk3* genes are viable and develop normally (Dong *et al.*, 1998; Yang *et al.*, 1997b; 1998). However, in a recent study, in which dual JNK knockouts were generated, it was found that the dual deficiency of JNK1 and JNK2 causes embryonic lethality due to severe dysregulation of apoptosis during brain development (Kuan *et al.*, 1999). Interestingly, depending on the brain region, the response is either pro-apoptotic or anti-apoptotic. This implies that JNK1 and JNK2 regulate the regional specificity of developmentally regulated apoptosis during brain morphogenesis. In *jnk1/jnk2* double knockout embryos, c-Jun is normally expressed and phosphorylated, presumably by JNK3, which further excludes c-Jun as a mediator of normal developmentally regulated apoptosis (Kuan *et al.*, 1999). Also in *Drosophila* genetic evidence for a role of JNK signaling in apoptosis in the context of an intact organism has been provided. It was shown that in the developing wing imbalanced BMP-signaling could cause JNK-mediated apoptosis (Adachi-Yamada *et al.*, 1999). It is not clear yet whether *Drosophila* Jun or Fos plays a role in this process.

c-Jun in death protection

In contrast to the findings discussed above, which support a role of JNK-Jun signaling in stimulating apoptosis, a growing amount of evidence implicates c-Jun in the protection of cells from stress-induced apoptosis. The most compelling evidence for c-Jun acting as an anti-apoptotic factor rather than an effector of apoptosis has again been obtained using c-Jun-deficient cells. In comparison to wild type cells, c-*jun*−/− fibroblasts have a greatly reduced capacity to escape apoptosis triggered by UV-induced cell stress. The anti-apoptotic function of c-Jun is phosphorylation dependent, since cells expressing the Ser63Ala, Ser73Ala mutant of c-Jun are not protected from apoptosis triggered by UV irradiation (Wisdom *et al.*, 1999). Consistent with the cell culture studies, the lack of c-Jun results in massive apoptosis of hepatoblasts and erythroblasts in developing mouse liver *in vivo* (Eferl *et al.*, 1999).

Concluding remarks

The AP-1 system has the capacity to funnel a great deal of complex information to a relatively simple promoter element comprised of only 7 or 8 base pairs. The dilemma that the cell faces is how to interpret the activation of AP-1 correctly and ensure that the initiated response, for example apoptosis or survival, is appropriate with regard to the extracellular information received.

Several mechanisms have been documented or suggested that could mediate the specific interpretation of JNK-Jun signaling by the cell. At the level of gene expression, the question is how AP-1 targets are selected in a cell type or context-dependent manner. Furthermore, one might ask whether there are quantitative, kinetic or qualitative changes in the way in which the transcription of relevant genes is regulated by AP-1 upon stimulation by different factors. This might lead the cell to a different fate, such as death or survival. All these parameters may be influenced by subtle alterations of the signaling properties within a cell. The effect on target gene activation or repression may be determined by the composition of AP-1 subunits in the cell, by other transcription factors that act in concert with AP-1 on target gene regulatory sequences, or by global effects on chromatin. The latter may be affected by the history of the cell or by extracellular signals (Thomson *et al.*, 1999). It should also be kept in mind that Jun and Fos proteins have several modes of action. In addition to their 'classical role' as TGACTCA-binding AP-1 transcription factors, they can influence gene activity by inhibitory interactions with other transcription factors, such as nuclear receptors or HLH proteins, at the protein level. The presence or absence, or the activation state of such interaction partners in a target cell might alter the quality of the AP-1 response. Finally, it is even conceivable that part of the complexity of the cellular response to AP-1 regulation may not depend on gene regulation at all. Preston *et al.* (1996) suggested that the Fos-induced apoptosis in Syrian hamster embryo cells is independent of protein synthesis.

Taken together, there are many variables that can influence the cell's receptiveness to and interpretation of JNK and AP-1 activation. Some of these variables are probably the activation state of known proteins, signaling pathways or chromatin domains. Others might be obscure and experimentally harder to grasp (to date!) and include cell- or signal-specific differences in signaling kinetics or topology. It will be a very important task to pull all these building blocks together in order to obtain a comprehensive picture of the mechanisms mediating context-specific signal interpretation by the cell.

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Differential regulation of c-Jun by ERK and JNK during PC12 cell differentiation

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The two MAP kinases JNK and ERK direct distinct cellular activities even though they share a number of common substrates, including several transcription factors. Here we have compared JNK and ERK signalling during PC12 cell differentiation and investigated how activation of c-Jun by the MAPKs contributes to this cellular response. Exposure to nerve growth factor, or expression of constitutively active MEK1—two treatments which cause differentiation of PC12 cells into a neuronal phenotype—result in activation of ERK-type MAP kinases and phosphorylation of c-Jun on several sites including Ser63 and Ser73. Constitutively activated c-Jun, which mimics the MAPK-phosphorylated form of the protein, can induce neuronal differentiation of PC12 cells independently of upstream signals. Conversely, expression of dominant-negative c-Jun^{bZIP} prevents neurite outgrowth induced by activated MEK1. Activation of MEKK1, which stimulates the JNK pathway, is not sufficient for PC12 cell differentiation but can induce apoptosis. However, neurite outgrowth is triggered when c-Jun is co-expressed with activated MEKK1 or SEK1. Consistently, MEK-induced ERK activation in PC12 cells induces c-Jun expression, while JNK signalling does not. Therefore, dual input of expression and phosphorylation of c-Jun provided by the ERK pathway is required to direct neuronal differentiation in PC12 cells.

Keywords: c-Jun/differentiation/MAP kinases/phosphorylation/signal transduction

Introduction

MAP kinase cascades are universal signal transduction modules that are evolutionarily conserved and used in a wide variety of biological response mechanisms. In vertebrates, at least three such pathways have been identified, which activate different MAP kinase classes, known as ERK, JNK and p38 (Treisman, 1996; Robinson and Cobb, 1997). Even though these signalling systems are built from evolutionarily related protein kinases, they convey distinct biological responses. Whereas ERK signalling is generally involved in the control of cell proliferation and differentiation, JNK and p38 signal transduction pathways mediate responses to various forms of cellular stress, such as damage repair mechanisms, cell growth arrest and cell death. The biological effects of MAP kinase signalling are executed by downstream phosphorylation substrates, most notably a number of signal-responsive transcription factors. A conceptual complication arose when it became evident that several of the MAP kinase-regulated transcription factors such as Elk1 and ATF-2 can serve as substrates for more than one MAP kinase and thus participate in different biological responses (Gupta *et al.*, 1995; Livingstone *et al.*, 1995; Van Dam *et al.*, 1995; Whitmarsh *et al.*, 1995, 1997; Price *et al.*, 1996). This raised the question of how the specificity of signal response is maintained, i.e. how distinct biological responses are mounted after kinases with similar or overlapping substrate specificity are activated.

The transcription factor c-Jun provides a useful model to study the complexity and specificity of signalling. c-Jun is an inducible transcription factor which directs changes of gene expression in response to multiple extracellular stimuli (Angel and Karin, 1991; Karin *et al.*, 1997). Transcription of c-*jun* mRNA rises after exposure of cells to a number of treatments including exposure to mitogens and various forms of stress. In addition to this transcriptional mode of regulation, c-Jun activity can also be modulated directly at the protein level. Most notable in this regard are regulatory phosphorylations occurring on Ser63 and Ser73, and Thr91 and/or Thr93 within the *trans*-activation domain (Pulverer *et al.*, 1991; Smeal *et al.*, 1992; Papavassiliou *et al.*, 1995). Phosphorylation of these residues results in the stabilization of c-Jun, as well as enhanced *trans*-activation and DNA-binding activity (Devary *et al.*, 1992; Radler-Pohl *et al.*, 1993; Papavassiliou *et al.*, 1995; Musti *et al.*, 1997). In certain cells, these phosphorylation events have been attributed to the MAP kinases ERK1 and ERK2 (Binetruy *et al.*, 1991; Pulverer *et al.*, 1991, 1993; Smeal *et al.*, 1991). ERKs are regulated by growth factors, neurotrophins and phorbol esters. The signal for ERK activation is relayed from the cell surface to the nucleus through a well-characterized signal transduction pathway involving activation of the small GTP-binding protein Ras, and a kinase cascade comprised of Raf, MEK and ERKs (Marshall, 1995; Treisman, 1996).

c-Jun is also a substrate for a related group of MAPKs, called stress-activated protein kinases (SAPKs), or Jun N-terminal kinases (JNKs) (Kyriakis and Avruch, 1996; Treisman, 1996). Exposure of cells to certain cytokines, protein synthesis inhibitors, or various forms of stress, triggers a kinase cascade leading to the activation of JNK, which can bind directly to and phosphorylate c-Jun (Hibi *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Sánchez *et al.*, 1994). Analogous to the ERK cascade, the JNK pathway involves the sequential activation of three kinases called MEKK, SEK and JNK, respectively (Kyriakis and Avruch, 1996; Robinson and Cobb, 1997).

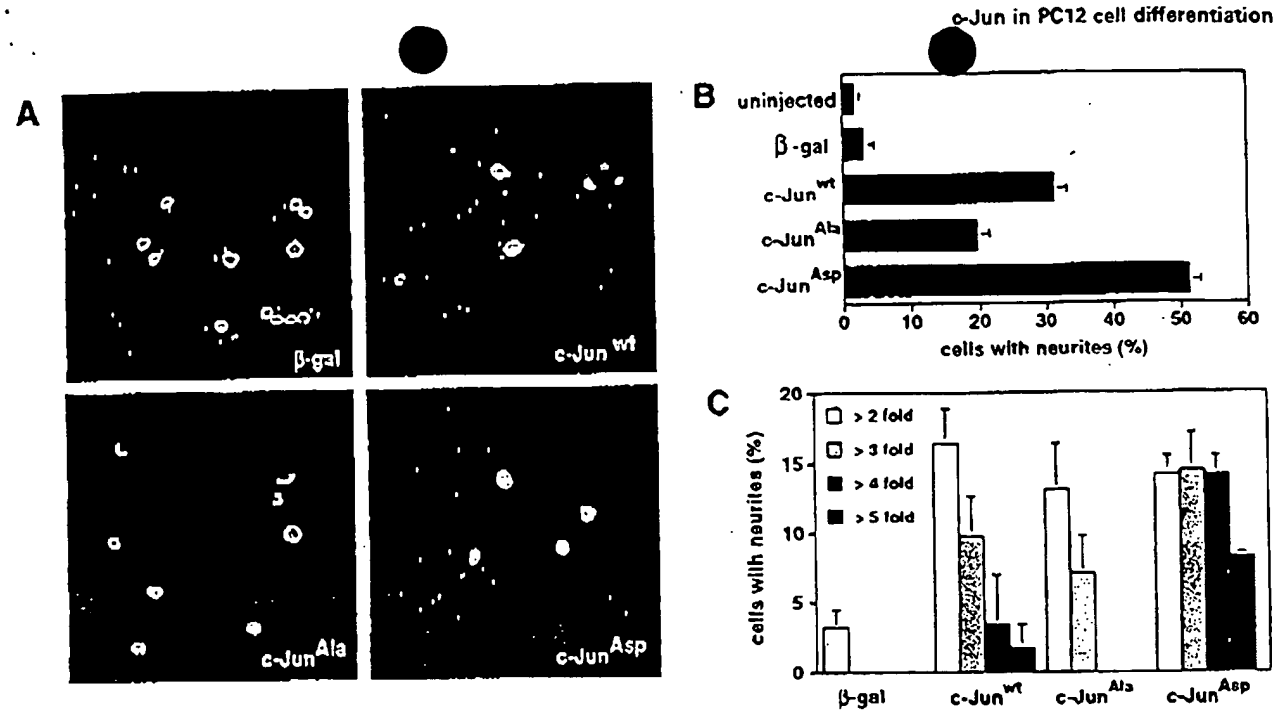

Fig. 1. Induction of neurite outgrowth in PC12 cells expressing c-Jun variants in which MAPK phosphorylation sites have been mutated. (A) Morphology of PC12 cells expressing c-Jun mutants. PC12 cells were microinjected with expression vectors for nuclear β-galactosidase, HA-tagged wild-type c-Jun, HA-c-Jun^{Ala} or HA-c-Jun^{Asp} as indicated. After 48 h, the cells were fixed and stained with anti-β-galactosidase or anti-HA antibodies. Injected cells were detected using FITC-labelled secondary antibodies (green), and the morphology of the cells was visualized by actin staining (red). Cells were examined under confocal microscopy. (B) Quantification of neurite outgrowth. The percentage of the cells with neurites exceeding twice the cell length among the microinjected (FITC-positive) cells is shown. (C) Length distribution of c-Jun-induced neurites. The percentage of cells with neurites exceeding 2-, 3-, 4- or 5-fold the cell length among the microinjected (FITC-positive) cells is shown. The data represent mean values ± SE of three separate experiments.

With the discovery of JNKs, it became apparent that these enzymes phosphorylate c-Jun more efficiently than ERKs *in vitro* (Dérijard *et al.*, 1994; Minden *et al.*, 1994a,b). Thus, the role of ERKs in the regulation of c-Jun activity has come into question.

The diversity of signals and signalling pathways that are directed toward c-Jun is also reflected in the biological responses, in which the transcription factors have been implicated. It was thought earlier that the main function of c-Jun is to transmit proliferative signals in a cell (Schütte *et al.*, 1989; Bos *et al.*, 1990; Castellazzi *et al.*, 1991; Lloyd *et al.*, 1991; Johnson *et al.*, 1993). Paradoxically, it has also been reported that c-Jun is involved in certain types of apoptotic cell death. For example, dominant-negative mutants of c-Jun, or antibodies against c-Jun were shown to protect neuronal cells from apoptosis induced by nerve growth factor (NGF) withdrawal, a treatment which activates the JNK and p38 kinase cascades (Estus *et al.*, 1994; Ham *et al.*, 1995; Xia *et al.*, 1995). Finally, Jun signalling can positively or negatively regulate differentiation in a number of systems (Bengal *et al.*, 1992; Treier *et al.*, 1995; Hou *et al.*, 1997; Kockel *et al.*, 1997; Riesgo-Escovar and Hafen, 1997). The exact molecular role that c-Jun plays in these different situations awaits clarification.

To examine the role of c-Jun as a target for ERK and JNK signalling, we performed studies in PC12 cells, a well-established experimental system in which the choice between a range of different biological signal responses, proliferation, neuronal differentiation and cell death, can be studied in tissue culture. NGF treatment causes differentiation into a sympathetic neuron-like cell. This coincides with the cessation of cell proliferation, neurite outgrowth and expression of immediate early genes, including c-*jun* and c-*fos*, as well as late response genes believed to function as determinants of neuronal differentiation (Sheng and Greenberg, 1990). The NGF response in PC12 cells requires activation of ERKs, since blocking the kinase cascade either by a specific inhibitor or by expression of dominant interfering mutants or antibodies against Ras or MEK1 inhibits the differentiation (Kremer *et al.*, 1991; Thomas *et al.*, 1992; Cowley *et al.*, 1994; Pang *et al.*, 1995). Conversely, constitutive activation of the ERKs by activated Raf, Ras or MEK1 induces differentiation (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Wood *et al.*, 1993; Cowley *et al.*, 1994). Here, we used the PC12 cell system to investigate if and how activation of c-Jun by phosphorylation contributes to neuronal differentiation of PC12 cells, and how the differential response to JNK and ERK activation might be mediated.

Results

Phosphorylated c-Jun induces neurite outgrowth in PC12 cells

To study the functional role of c-Jun phosphorylation in PC12 cell differentiation, plasmids encoding various c-Jun derivatives in which previously identified MAPK substrate residues had been modified, were introduced into PC12 cells by microinjection. The expression of the HA-epitope-tagged c-Jun derivatives in recipient cells was monitored by immunostaining with anti-HA antibodies (Figure 1A).

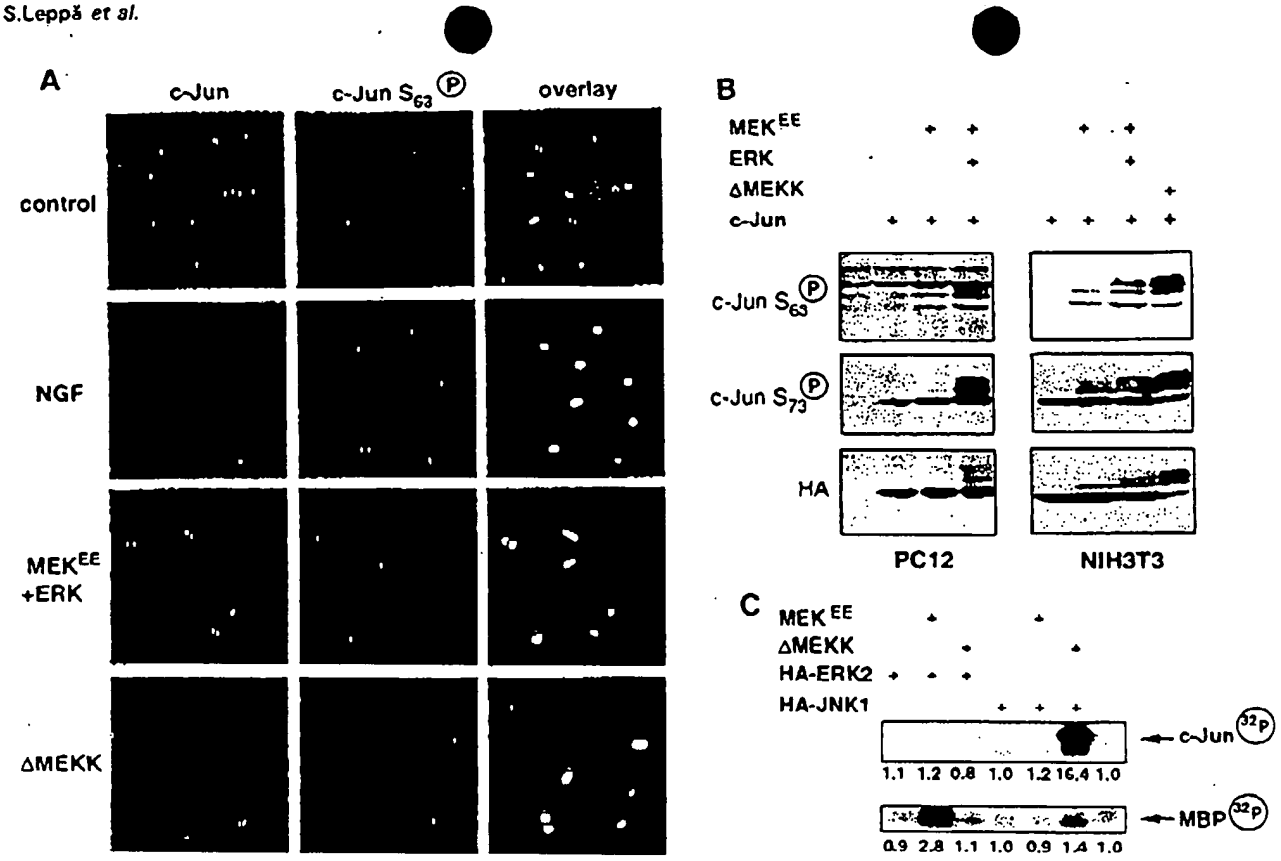

Fig. 2. Phosphorylation of c-Jun in response to NGF and activated MEK1. (A) Phosphorylation of c-Jun on Ser63 in PC12 cells. PC12 cells were injected with expression vectors for myc-tagged c-Jun alone, or together with vectors coding for activated MEK1 (MEK^{EE}) and ERK, or activated MEKK (ΔMEKK). NGF treatment was carried out for 60 min at 24 h post-injection. Doublestaining was performed with anti-myc antibody to detect the injected cells, and with anti-phospho-c-Jun to stain c-Jun phosphorylated on Ser63. These antibodies were chosen because the anti-c-Jun Ser73 phosphate antibodies used in the immunoblots were not suitable for immunostaining. Nuclei expressing myc–c-Jun appear red (left panel) and the phosphorylated form of c-Jun is visualized in green (middle panel). Note that a yellow colour in the overlay (right panel) indicates a high stoichiometry of c-Jun phosphorylation. (B) c-Jun is phosphorylated in response to ERK activation in PC12 and NIH 3T3 cells. HA-tagged c-Jun was expressed in PC12 or NIH 3T3 cells alone or together with MEK^{EE} and ERK2, or ΔMEKK as indicated. Cells were harvested 36 h post-transfection, and whole-cell extracts were analysed by SDS–PAGE and immunoblotting using antibodies against phosphorylated forms of c-Jun on Ser63 (top), Ser73 (middle), or an antibody against HA-epitope (bottom). (C) Specificity of JNK and ERK activation by ΔMEKK and MEK^{EE}, respectively. HA-tagged ERK or JNK was expressed in PC12 cells alone or together with MEK^{EE} or ΔMEKK (as a positive control) as indicated. The cells were harvested 36 h post-transfection, and the lysates were immunoprecipitated using anti-HA antibody. Immunocomplex kinase assay was performed using GST–c-Jun (amino acids 5–105; top) or myelin basic protein (MBP; bottom) as a substrate. The positions of GST–c-Jun and MBP are shown. The numbers below each lane indicate fold induction of kinase activity relative to the value measured in mock-transfected cells, as determined by PhosphorImager scanning.

In $c\text{-}Jun^{Asp}$, potential MAPK phosphorylation sites, including Ser63 and Ser73, and Thr91 and Thr93; have been replaced by phosphate-mimicking aspartic acid residues (Treier *et al.*, 1995). This 'gain of function' mutant acts like the active phosphoprotein in several assays (Papavassiliou *et al.*, 1995; Treier *et al.*, 1995; Musti *et al.*, 1997). Expression of $c\text{-}Jun^{Asp}$ caused the development of long neurites in >50% of microinjected PC12 cells (Figure 1B). If the concentration of the Jun^{Asp} expression vector was titrated down from the standard concentration of 50 μg/ml in the injected solution to 2 μg/ml, significant neurite outgrowth was still detected (data not shown), indicating that moderate overexpression was sufficient to elicit the described effects. In control experiments, $c\text{-}Jun^{Ala}$ (a mutant which cannot be phosphorylated by MAPKs) and $c\text{-}Jun^{wt}$ caused flattening of the cells, increased cell diameter and only moderate neurite formation (Figure 1B). The cells developed fewer and notably shorter neurites as compared with those injected with $c\text{-}Jun^{Asp}$ (Figure 1C). Control cells injected with an expression vector for nuclear β-galactosidase were not induced to differentiate. We conclude that phosphorylation of c-Jun plays an important role in directing PC12 cells towards a neuronal differentiation pathway.

c-Jun is a downstream target of the ERK pathway in PC12 cells

Since the results shown in Figure 1 imply that activation of c-Jun by phosphorylation, as mimicked by $c\text{-}Jun^{Asp}$ expression, is sufficient to cause PC12 cell differentiation, we next examined whether differentiation in response to NGF involves the same mechanism. Thus, the phosphorylation state of c-Jun upon NGF treatment was determined. PC12 cells expressing $c\text{-}Jun^{wt}$ were treated with NGF, and immunostained with an antibody (anti-c-Jun $Ser_{63}P$) that specifically recognizes the Ser63-phosphorylated form of c-Jun but not c-Jun that is unphosphorylated at this site. Figure 2A shows that Ser63-phosphorylated c-Jun was hardly detectable in unstimulated cells. However, NGF treatment induced prominent c-Jun phosphorylation. Thus,

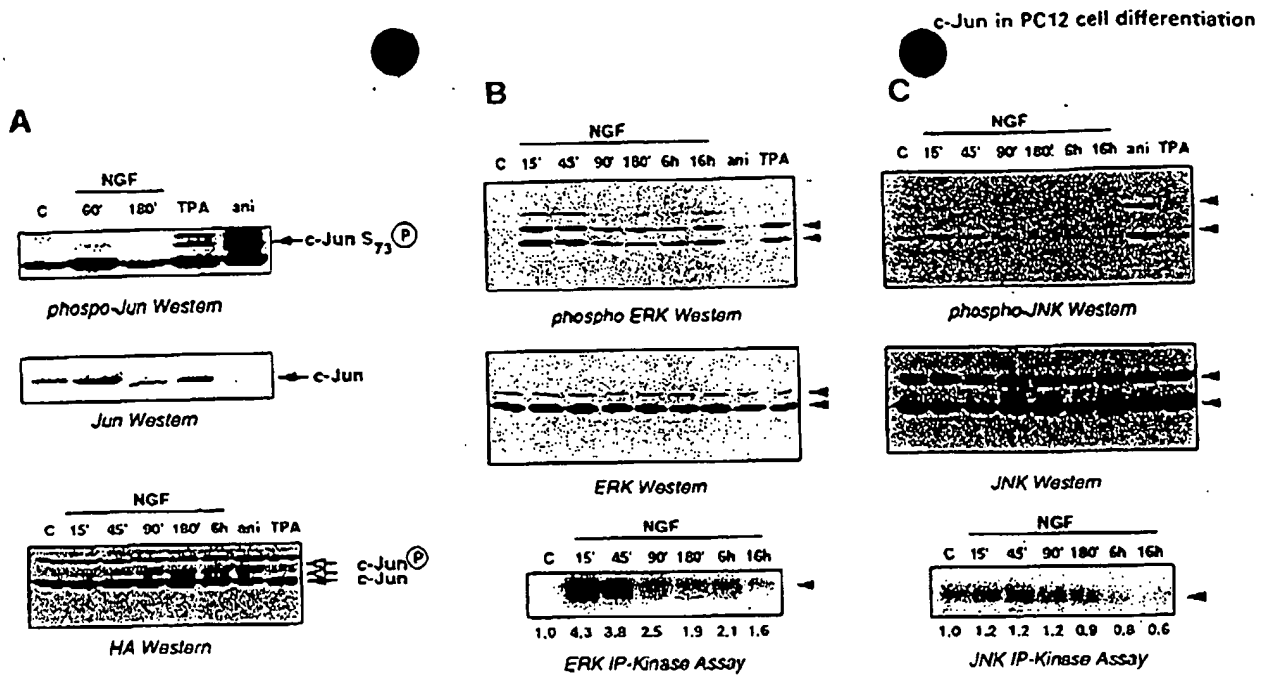

Fig. 3. NGF-mediated phosphorylation of c-Jun, ERKs and JNKs in PC12 cells. (A) Expression and phosphorylation of c-Jun in response to NGF. Top: nuclear extracts from PC12 cells treated with NGF for the indicated periods of time, with TPA for 1 h, or with anisomycin (ani) for 30 min, were subjected to SDS–PAGE and immunoblot analysis. Endogenous c-Jun was detected using an antibody against phosphorylated forms of c-Jun on Ser73 or an antibody against bacterially expressed c-Jun. Bottom: HA-tagged c-Jun was expressed in PC12 cells. At 36 h post-transfection, cells were starved for 6 h, and subsequently stimulated with NGF, anisomycin (ani) or TPA. Whole-cell extracts were prepared and analysed by SDS–PAGE and immunoblotting using anti-HA antibody. The position of phosphorylated and non-phosphorylated c-Jun is indicated by open and closed arrows, respectively. (B) Activation of ERKs in response to NGF. Top: whole-cell extracts from PC12 cells treated with NGF for the indicated periods of time, with anisomycin for 30 min, or with TPA for 1 h were subjected to SDS–PAGE and immunoblot analysis using an antibody against dual-phosphorylated ERKs. The lower panel shows an identical filter probed with anti-ERK2 antibody. The positions of ERK1 (upper band) and ERK2 (lower band) are indicated by arrowheads. Bottom: lysates from control cells (C) and cells treated with NGF for the indicated periods of time were immunoprecipitated using anti-ERK2 antibody. Immunocomplex kinase assay was performed using MBP as a substrate. The position of MBP is shown. The numbers below each lane indicate fold induction of ERK activity relative to the control level. (C) Activation of JNKs in response to NGF. Top: JNK activation upon NGF treatment was analysed using an antibody against activated JNKs, as described for (B). The lower panel shows an identical filter probed with anti-JNK1 antibody. The positions of JNK1 (lower band) and JNK2 (upper band) are shown. Bottom: lysates from control and NGF-treated cells were immunoprecipitated using anti-JNK1 antibody. Immunocomplex kinase assay was performed using GST–c-Jun (amino acids 5–105) as a substrate. The position of GST–c-Jun is shown. The numbers below each lane indicate fold induction of JNK activity relative to the control level.

NGF induces phosphorylation of c-Jun in PC12 cells which, according to the data shown in Figure 1, would be sufficient to initiate neuronal differentiation.

We next investigated the pathway through which the NGF signal is transmitted to the PC12 cell nucleus to induce c-Jun phosphorylation. It has previously been shown that NGF stimulates a signal transduction pathway that culminates in the activation of ERKs. Furthermore, PC12 cells differentiate in response to expression of activated components of this pathway, such as Ras, Raf and MEK1 (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Wood *et al.*, 1993; Cowley *et al.*, 1994). Hence, we first tested whether MEK1, a MAPK kinase specific for ERKs, could be a mediator between NGF and c-Jun phosphorylation. When c-Jun was co-expressed with a constitutively activated form of MEK1 (MEK^{EE}; see Materials and methods) and ERK2, intense nuclear immunostaining was detected with the anti-c-Jun $Ser_{63}P$ antibody (Figure 2A). Under our experimental conditions, expression of MEK^{EE} or ERK2 alone did not result in strong phosphorylation (Figure 2B and C; data not shown), indicating that ERK protein concentration is a limiting factor for c-Jun phosphorylation in the cells. Prominent c-Jun phosphorylation was also detected in cells in which c-Jun was co-expressed with an activated JNK pathway component, such as the constitutively active MEKK1 (ΔMEKK), comprised of a 672-residue C-terminal fragment of the molecule (Whitmarsh *et al.*, 1995) (Figure 2A), or activated SEK plus JNK (data not shown). To corroborate these findings, we performed immunoblotting experiments with extracts from transiently transfected PC12 cells using phospho-specific antibodies or antibodies that are not sensitive to the phosphorylation state of c-Jun. In agreement with the immunostaining results shown in Figure 2A, we find that co-expression with MEK^{EE} and ERK2 results in significant phosphorylation of c-Jun on Ser63 and Ser73. Qualitatively similar results were obtained when the same experiment was performed in NIH 3T3 cells, and after co-transfection with ΔMEKK. Taken together, these results indicate that, in both PC12 and NIH 3T3 cells, c-Jun can be phosphorylated on Ser63 and Ser73 in an ERK- or a JNK-dependent manner. To monitor directly the specificity of MAPK activation under our assay conditions we performed immunocomplex kinase assays (Figure 2C). This experiment shows that, as expected, MEK^{EE} stimulated the kinase activity of HA-tagged ERK2 measured on MBP as a substrate. Importantly, no significant ERK activation was observed after co-expression with ΔMEKK, indicating that under our experimental conditions there is no cross-talk between the JNK and ERK signalling pathway. This finding further supports our conclusion that c-Jun is a target to ERK

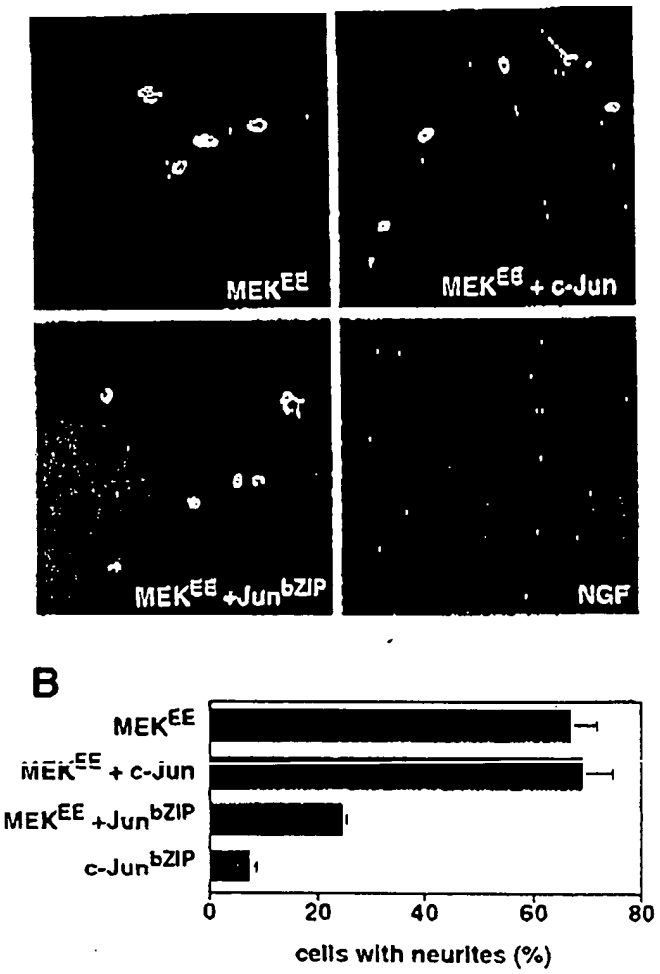

Fig. 4. Dominant-negative Jun inhibits MEK-induced neurite outgrowth in PC12 cells. (A) Morphology of PC12 cells expressing activated MEK and c-Jun. PC12 cells were injected with expression vectors for MEK^{EE} alone, MEK^{EE} together with c-Jun^{wt}, or MEK^{EE} with c-Jun^{bZIP}, as indicated. Nuclear β-galactosidase was co-expressed to mark the injected cells. After 48 h, the cells were fixed and stained with anti-β-galactosidase (green) and TRITC-phalloidin (red). NGF-treated cells stained with TRITC-phalloidin are shown as a control. (B) Quantification of neurite outgrowth. The percentage of the cells with neurites exceeding twice the cell length among the microinjected (FITC-positive) cells is shown. The data shown are the mean values ± SE of two separate experiments.

phosphorylation in PC12 cells. In contrast, ΔMEKK specifically activated JNK and had no effect on ERK activity. Like others (Minden *et al.*, 1994a,b) we observed no phosphorylation of c-Jun by ERK *in vitro*. This negative result may be explained by a requirement for further factors in the cell that facilitate the phosphorylation of c-Jun by ERK.

To gain further insight into the relationship between ERK, JNK and c-Jun activation in PC12 cell differentiation, we analysed the phosphorylation state and hence the activity of these proteins over a time course following NGF addition. The top panel of Figure 3A shows that both the expression levels and the phosphorylation of endogenous c-Jun protein is increased after NGF treatment of PC12 cells. The expression of c-Jun directed from a transfected CMV vector is not stimulated by NGF (Figure 3A, bottom panel) and thus permits assessment of NGF-dependent c-Jun phosphorylation over time at constant protein levels. This experiment reveals a kinase activity present in PC12 cells for hours after NGF induction that can specifically phosphorylate c-Jun. ERKs are good candidates for such an activity, as the strong and sustained activation of ERK1 and ERK2 (Figure 3B) correlates well with the persistence of phosphorylated forms of c-Jun (Figure 3A). In contrast, we observed only a weak and transient JNK1 activation after 15 min of NGF treatment, whereas JNK2 activity was not increased or even slightly reduced (Figure 3C). Consistently, when we measured JNK activation by immunocomplex kinase assay, we found that the basal levels of JNK activity were reduced upon exposure of cells to NGF (Figure 3C, bottom panel). Thus, while a contribution of JNK to c-Jun phosphorylation cannot be completely excluded, the kinetics and amplitude of ERK activity is more compatible with a major function of the latter class of MAPKs in the phosphorylation of c-Jun upon NGF treatment.

If c-Jun is an essential downstream component of the MEK1/ERK-mediated differentiation in PC12 cells, a dominant-negative form of c-Jun would be expected to interfere with this process. Conversely, wild-type c-Jun might be anticipated to enhance neurite outgrowth. Figure 4 shows that co-expression of c-Jun^{wt} with MEK^{EE} did not markedly increase the number of cells forming neurites, yet the neurites were longer, as compared with cells expressing MEK^{EE} alone. Similar results were obtained after co-expressing MEK^{EE} and ERK2 (data not shown). In contrast, co-expression of c-Jun^{bZIP}, a truncated, dominant-negative form of Jun, caused marked inhibition of MEK1 and ERK2-induced neurite outgrowth. Expression of c-Jun^{bZIP} alone did not result in any cellular responses (Figure 4B). These results therefore provide evidence that the MAPK/ERK pathway can trigger c-Jun phosphorylation, and that this event is critical for PC12 cell differentiation in response to NGF.

JNK can induce neuronal differentiation only if c-Jun is co-expressed

Next, we asked whether JNK, which can also phosphorylate the Ser63 and Ser73 residues of c-Jun, may elicit PC12 cell differentiation when activated. In the JNK signalling pathway, MEKK1 phosphorylates and activates SEK1, also called MKK4, which in turn activates JNK by phosphorylating its regulatory Tyr and Thr residues (Lange-Carter *et al.*, 1993; Dérijard *et al.*, 1994; Kyriakis *et al.*, 1994; Sánchez *et al.*, 1994; Yan *et al.*, 1994; Lin *et al.*, 1995). Two components of the JNK pathway, ΔMEKK and partially active SEK1 (SEK^{ED}), in which the regulatory phosphorylation sites have been substituted by glutamic and aspartic acid residues (J.Woodgett, personal communication) were used in the experiments. Immunoblot analysis of transiently transfected NIH 3T3 cells (Figure 5A) indicated that SEK^{ED} could induce phosphorylation of c-Jun^{wt} *in vivo*, albeit to a lesser extent than MEK^{EE}. Phosphorylation of c-Jun was strongest when c-Jun was co-expressed with ΔMEKK. The same assay was also performed using c-Jun^{Ala} as a substrate for these kinases. c-Jun^{Ala} was not recognized by anti-c-Jun Ser63

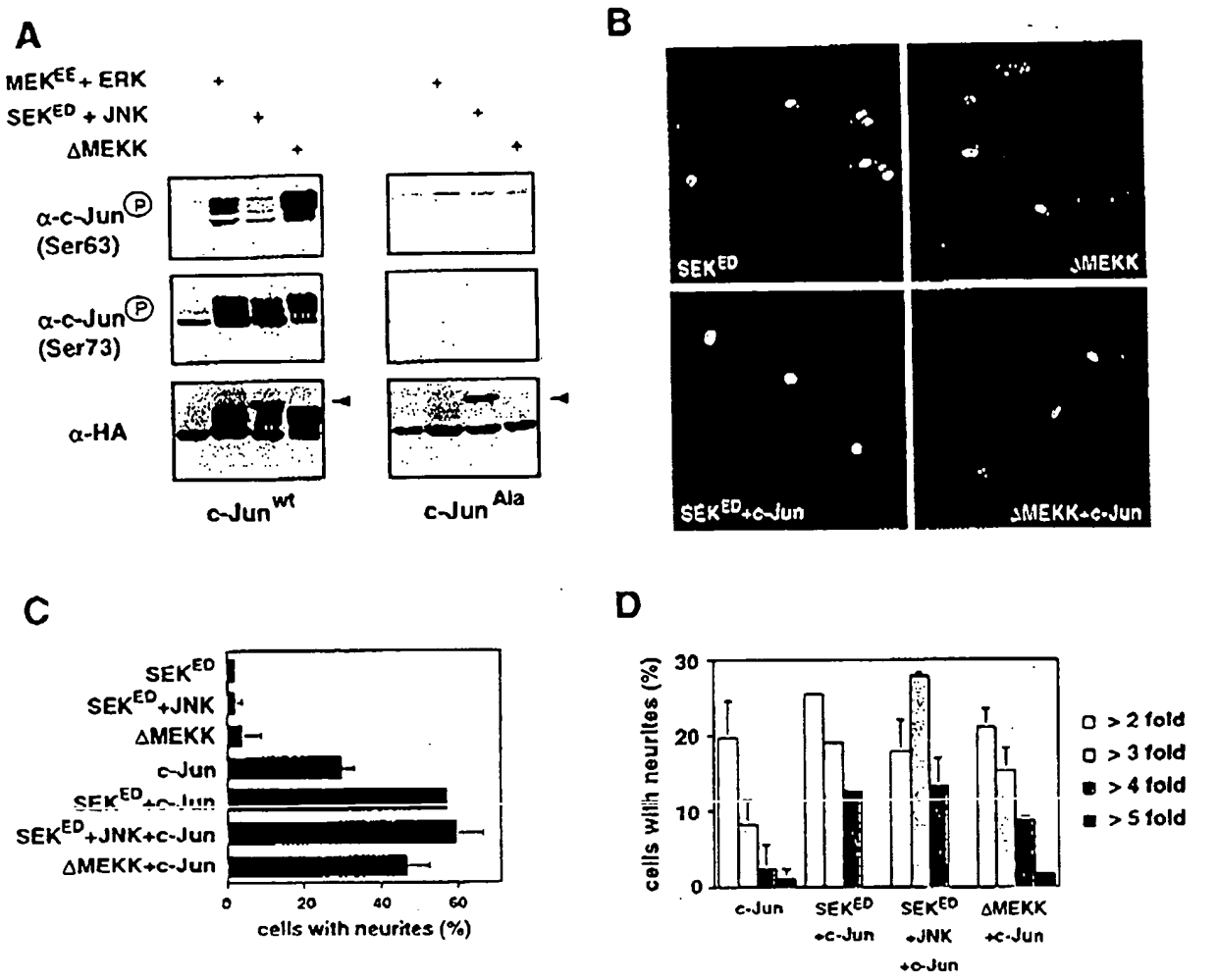

Fig. 5. Activation of MEKK or SEK can mediate PC12 cell differentiation in the presence of c-Jun. (A) c-Jun is phosphorylated in response to activated MEK, SEK and MEKK in NIH 3T3 cells. c-Jun^{wt} or c-Jun^{Ala} were expressed in NIH 3T3 cells alone or with MEK^{EE}, ERK, SEK^{ED}, JNK or ΔMEKK, as indicated. Cells were harvested 24 h post-transfection. Whole-cell extracts were assayed for c-Jun phosphorylation using SDS-PAGE and immunoblotting with antibodies against c-Jun phosphorylated on Ser63 (top) or Ser73 (middle), or anti-HA antibody (bottom). The arrowhead indicates JNK that is also HA-tagged. (B) Morphology of PC12 cells expressing activated components of the JNK pathway. PC12 cells were injected with expression vectors for SEK^{ED} or ΔMEKK in the absence or presence of a plasmid coding for c-Jun^{wt}, as indicated. Nuclear β-galactosidase was co-expressed to mark the injected cells. The cells were fixed after 40 h and stained with anti-β-galactosidase (green) and TRITC-phalloidin (red). (C, D) Quantification of neurite outgrowth and length distribution of neurites were performed as in Figure 1B and C, respectively. The data shown are mean values ± SE of two separate experiments.

or Ser73 phosphate antibodies, verifying the specificity of these antibodies.

The ability of the activated components of the JNK pathway to elicit PC12 cell differentiation responses was examined using the microinjection assay (Figure 5B). In contrast to MEK^{EE} (Figure 4), neither SEK^{ED} nor ΔMEKK induced neurite outgrowth when expressed in PC12 cells, which further indicates that activation of JNK pathway is not sufficient to induce PC12 cell differentiation. However, consistent with previously published results (Xia *et al.*, 1995), we found that ΔMEKK-expressing cells underwent apoptosis. PC12 cells expressing SEK^{ED} displayed neither differentiation nor apoptosis. If, however, SEK^{ED} or ΔMEKK were expressed along with c-Jun^{wt}, marked neurite outgrowth ensued (Figure 5B and C). The fraction of differentiating cells, as well as the average length of the appearing neurites, was significantly increased as compared with cells that had received c-Jun^{wt} alone (Figure 5C and D). Interestingly, the apoptotic effect of ΔMEKK was suppressed when c-Jun was co-expressed. The basis for this is unclear and subject to further investigation. The cooperation between JNK and c-Jun in the induction of neurite outgrowth suggests that c-Jun phosphorylation is sufficient to induce PC12 cell differentiation, regardless of whether it is mediated by MEK-induced ERK activity or through activation of JNK.

ERK- but not JNK-activation induces c-Jun expression in PC12 cells

An attractive hypothesis to explain the above results poses that PC12 cell differentiation requires two events, namely the induction of c-Jun synthesis and c-Jun phosphorylation. Whereas both ERK and JNK can catalyse phosphorylation of the relevant sites in c-Jun, only the former can stimulate c-Jun expression in PC12 cells efficiently (Figure 7). According to this model, JNK activation would thus not be sufficient to stimulate differentiation, unless c-Jun is provided *in trans*. To test this idea, we investigated

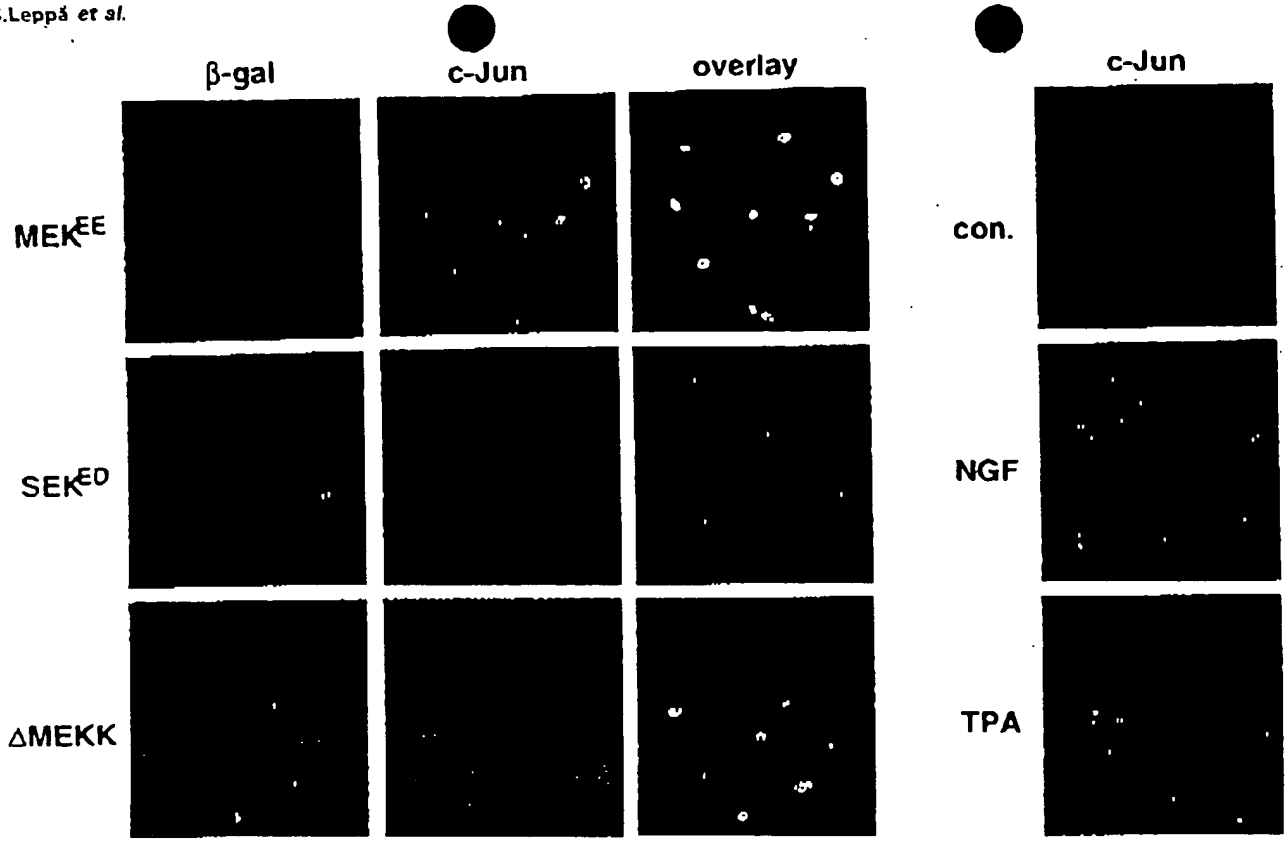

Fig. 6. c-Jun expression is induced by NGF and activated MEK, but not by activated MEKK and SEK. Left: expression of endogenous c-Jun in PC12 cells in response to activated MEK, MEKK and SEK. PC12 cells were injected with expression vectors coding for MEK^{EE}, ΔMEKK or SEK^{ED}. Nuclear β-galactosidase was co-expressed to mark the injected cells. The cells were fixed after 16 h and double stained with anti-β-galactosidase (red) and anti-c-Jun (green) antibodies. Note that a yellow colour in the overlay indicates prominent c-Jun immunoreactivity. Right: expression of endogenous c-Jun in response to NGF. Cells were starved for 16 h and subsequently treated with NGF and TPA (as a positive control) for 1 h. After fixation, the cells were stained with anti-Jun antibody.

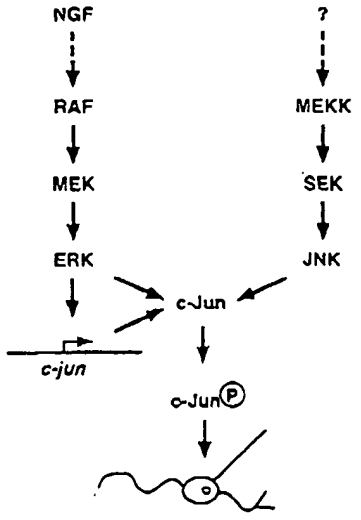

Fig. 7. Model of signalling to c-Jun in undifferentiated PC12 cells. Two MAPK cascades converge on c-Jun. The ERK pathway regulates c-Jun expression and phosphorylation during neuronal differentiation of PC12 cell. If c-Jun expression levels are increased JNK pathway can also mediate differentiation response.

whether endogenous c-Jun expression can be induced by specifically activating either the ERK or the JNK pathway. MEK^{EE}, SEK^{ED} or ΔMEKK were expressed in PC12 cells, and endogenous c-Jun expression was examined by immunostaining (Figure 6). Interestingly, expression of MEK^{EE} caused a significant increase in c-Jun expression similar to the one seen in NGF- or TPA-treated PC12 cells (Figure 6, right panel). In contrast, ΔMEKK, which effectively activates JNK, resulted in only marginal c-Jun induction. When PC12 cells were injected with expression plasmid for SEK^{ED}, c-Jun expression was not detectably induced. Taken together, these results establish a biologically relevant difference between the ERK and the JNK-signalling in the regulation of c-Jun activation.

Discussion

Several lines of evidence presented here suggest that c-Jun can act as a substrate for ERK phosphorylation in at least two different cell types, and that in the NGF response, ERK-mediated activation of c-Jun directs PC12 cells towards neuronal differentiation. First, analogous to activated components of the Ras/MAPK pathway such as Ras, Raf or MEK1 (Bar-Sagi and Feramisco, 1985; Noda *et al.*, 1985; Wood *et al.*, 1993; Cowley *et al.*, 1994), expression of $c\text{-}Jun^{Asp}$ in PC12 cells induced marked neurite outgrowth. Secondly, NGF treatment of PC12 cells induced sustained activation of ERKs and phosphorylation of c-Jun. A transient small increase in JNK1 activity after NGF exposure does not match the kinetics of c-Jun phosphorylation. Thirdly, expression of constitutively active MEK and ERK, which has been shown previously to lead to PC12 cell differentiation (Cowley *et al.*, 1994), resulted in prominent c-Jun expression and phosphorylation on Ser63 and Ser73, but not JNK activation. Fourthly,

expression of c-Jun potentiated differentiation of PC12 cells induced by MEK1, whereas dominant-negative mutants of c-Jun inhibited it.

Thus, while ERKs are less effective kinases of c-Jun *in vitro*, as compared with JNKs, phosphorylation of c-Jun by ERKs appears to mediate signal responses *in vivo*, at least during PC12 cell differentiation. This is consistent with our recent findings in *Drosophila*, which indicate that Jun can act as an effector of both JNK and ERK pathways during development of this organism (Peverali *et al.*, 1996; Kockel *et al.*, 1997). Nevertheless, JNKs can phosphorylate c-Jun more efficiently than ERKs on the sites which, according to our mutant analysis, are critical for PC12 cell differentiation. Considering this—and our finding that phosphorylation of c-Jun on these sites is sufficient to direct PC12 cells along a path of neuronal differentiation—one might predict that activation of the JNK pathway would also induce PC12 cells to differentiate. However, this is not the case. Stimulation of JNK activity either by activated forms of MEKK or SEK does not trigger neurite formation in the way that it was seen when the ERK pathway was stimulated (by MEK^{EE} or by NGF). Instead, consistent with observations by others (Xia *et al.*, 1995; Lassignal Johnson *et al.*, 1996), ΔMEKK induced apoptosis in undifferentiated PC12 cells. In this situation, c-Jun seems not to be involved, but conversely counteracts apoptosis and induces differentiation when provided in addition. Since c-Jun itself has also been implicated in apoptosis in some circumstances (Ham *et al.*, 1995; Xia *et al.*, 1995; Watson *et al.*, 1998), it seems clear that the effects of c-Jun on cellular responses depend on the cell type and the context of regulatory inputs that the cell is receiving.

Our results show that a JNK activating signal could promote neuronal differentiation in PC12 cells only when c-Jun was provided in addition. These data suggest a model in which activation of the ERK pathway in PC12 cells results in stimulation of both c-Jun synthesis and c-Jun phosphorylation, whereas the JNK pathway triggers phosphorylation only (Figure 7). Indeed, we could show that activation of ERK pathway, but not JNK, induced prominent c-Jun expression in PC12 cells. This is consistent with recent data reporting that activation of JNK is not sufficient to activate the c-*jun* promoter in fibroblasts (Hazzalin *et al.*, 1996). Providing c-Jun exogenously, however, will turn the JNK activation into a differentiation signal. According to this model, the specificity of signal response is not based on a qualitative or quantitative difference in the way ERKs or JNKs phosphorylate Jun (e.g. in terms of kinetics or phosphorylation site preference). Phosphorylation of c-Jun by either kinase can promote PC12 cell differentiation. Based on the data presented here, we favour a combinatorial model in which a dual input on the level of transcription and phosphorylation of c-Jun is required to start a programme of neuronal differentiation in PC12 cells. There may also be situations where simultaneous activation of ERK and JNK act synergistically to elicit a response that is distinct from the response to the activation of either pathway alone.

Materials and methods

Plasmids

Plasmids for mammalian cell expression of CMV-driven epitope-tagged $c\text{-}Jun^{wt}$, $c\text{-}Jun^{Ala}$, $c\text{-}Jun^{Asp}$ and nuclear β-galactosidase have been described (Treier *et al.*, 1994, 1995). CMV-driven expression vector for HA-tagged dominant-negative form of c-Jun ($c\text{-}Jun^{bZIP}$) was constructed by deleting amino acids 25–181 from the sequence. Bacterial pGEX2T expression vector containing GST–c-Jun (kindly provided by F.A.Peverali) was constructed by PCR amplification of sequences containing amino acids 5–105. Constructs for mammalian pEXV expression vectors for constitutively active MEK1 and ERK2myc (Cowley *et al.*, 1994) were provided by C.Marshall; the construct for mammalian expression of constitutively active MEKK1 (Whitmarsh *et al.*, 1995) was provided by R.J.Davis; the construct for mammalian expression for HA-tagged JNK (Coso *et al.*, 1995) was provided by S.Gutkind; the construct for mammalian expression for HA-tagged ERK2 was provided by C.J.Der; and the construct for mammalian expression for partially active SEK was provided by J.Woodgett.

Cell culture and transfections

Rat phaeochromocytoma PC12 cells were routinely cultured on collagen-coated dishes in a humidified 7.5% CO_2 atmosphere at 37°C in DMEM, supplemented with 10% horse serum (HS) and 5% fetal calf serum (FCS). Transient transfection into PC12 cells was done with Lipofectamine according to the manufacturer's instructions (Gibco-BRL). Mouse NIH 3T3 cells were cultured in DMEM with 10% calf serum (CS). Transfection was performed using the calcium phosphate method (Graham and van der Eb, 1973). Cells were harvested 24–36 h after transfection.

Microinjection

For microinjection, cells were seeded on laminin-coated plastic plates (20 μg/ml mouse EHS-laminin; Boehringer) to provide better adhesion and facilitate neurite outgrowth. Microinjections were performed on an automated injection system using a Zeiss inverted microscope. All plasmids were injected into the nucleus at a concentration of 50 μg/ml unless otherwise stated. 100–150 cells were injected per experiment.

Immunostaining

Cells were fixed with 2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), washed with PBS and permeabilized with 0.1% Triton X-100 in PBS on ice. Blocking with 1% bovine serum albumin (BSA) in PBS for 30 min, and incubations with primary antibodies in 1% BSA–PBS for 1 h, were done at room temperature (RT). Antibodies included a monoclonal antibody (mAb) against β-galactosidase (Promega), a mAb against HA-epitope (clone 12CA5), a mAb against myc-epitope (clone 9E10), a polyclonal antibody against c-Jun (Bohmann and Tjian, 1989), and a polyclonal antibody against phosphorylated c-Jun at position Ser63 (New England Biolabs). After several washes, bound antibodies were detected using FITC- and Texas red-conjugated secondary antibodies (Dianova) for 1 h at RT. The morphology of the cells was visualized using TRITC-labelled phalloidin (Sigma). Cells were further washed extensively with PBS, and Hoechst dye 33258 (Sigma) was included in the last wash to visualize the nuclei. Finally, the cells were mounted under a coverslip using Mowiol. Samples were examined using a Zeiss LSM410 confocal imaging system. For quantification of neurite outgrowth, the cells forming neurites longer than twice the diameter of the cell body were defined as positive.

Western blot analysis

Whole-cell extracts were prepared by lysing the cells directly in SDS sample buffer. Nuclear extracts were obtained by extracting the cells with hypotonic lysis buffer (10 mM Tris pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.2 mM Na_3VO_4, 50 μM NaF, 2 mM DTT, 0.5% NP-40), followed by solubilization of nuclei into SDS sample buffer. After sonication, protein samples (10–20 μg) were separated on SDS–polyacrylamide gels and electroblotted onto nitrocellulose filters. Immunoblotting was performed using a mAb against HA-epitope, polyclonal antibodies against phospho-c-Jun (anti-c-Jun phosphorylated on Ser63 or Ser73; New England Biolabs), polyclonal antibody against c-Jun (Bohmann and Tjian, 1989), and polyclonal antibodies against activated phospho-ERKs and phospho-JNKs (Promega). ERK1/2 and JNK1/2 were detected by polyclonal antibodies C-14 and C-17, respectively (Santa Cruz). HRP-conjugated secondary antibodies were purchased from Dianova. The blots were developed with an enhanced chemiluminescence method (ECL, Amersham).

In vitro kinase assays

The cells were washed with PBS and solubilized in lysis buffer (25 mM HEPES–NaOH pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 5 mM EDTA, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM

Na_3VO_4). Transiently transfected HA-tagged JNK1 was immunoprecipitated for 1 h at 4°C using monoclonal anti-HA (12CA5) antibody. Immunocomplexes were coupled to protein-A–Sepharose beads for an additional 1 h at 4°C and washed four times with dilution buffer (25 mM HEPES–NaOH pH 7.5, 5 mM EDTA, 5 mM EGTA, 25 mM β-glycerophosphate, 0.1 mM Na_3VO_4), followed by one wash with kinase buffer (50 mM HEPES–NaOH pH 7.5, 10 mM $MgCl_2$, 1 mM DTT, 25 mM β-glycerophosphate, 1 mM Na_3VO_4). Kinase reactions were performed in the presence of 2 μCi of [γ-^{32}P]ATP for 20 min at 30°C using myelin basic protein (MBP) or GST–c-Jun (5–105) as a substrate. Phosphorylated proteins were analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. The intensities of the radioactive signals were quantitated with a PhosphorImager (Molecular Dynamics).

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